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| <p>(54) Title: REAGENT MIXTURES FOR GLUCOSE ASSAY</p> <p>(57) Abstract</p> <p>The present invention relates to a test reagent mixture composition comprising the enzymes glucose oxidase and peroxidase and a chromogen which interacts with the hydrogen peroxide from the oxidation of the blood glucose by the glucose oxidase, characterised in that the glucose oxidase and the peroxidase are present in proportions which provide from 300 to 700 International Units (IUs) of glucose oxidase and at least 20 International Units of peroxidase and in that the chromogen is present in an amount which provides from 12 to 20 grams of active chromogen per 500,000 International Units of glucose oxidase. Preferably, the composition is put up in a low molecular weight gelatin matrix and is impregnated into a micro-porous carrier membrane.</p> |           |  |

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## REAGENT MIXTURES FOR GLUCOSE ASSAY

The present invention relates to a reagent mixture, notably to a mixture of analytical reagents in a carrier gel which provides enhanced consistency of the colour generated with the elapse of time.

BACKGROUND TO THE INVENTION:

- 10 Blood samples are often assessed for the amount of glucose or some other constituent therein by reacting the blood with one or more reagents carried on a test stick or pad so as to develop a colour which can be observed by an operator. For example, a reagent pad can contain the enzymes glucose oxidase and peroxidase and o-tolidine as the chromogen which turns blue as the glucose oxidase oxidises glucose in the blood sample to gluconic acid and hydrogen peroxide. The hydrogen peroxide reacts in the presence of the peroxidase with the o-tolidine to give a blue colour whose intensity depends upon the amount of hydrogen peroxide released and hence the amount of glucose in the blood. The reagents are usually put up in a gel matrix, for example of a natural gel, for example a gelatin, or of a synthetic polymer, for example a polyvinylpyrrolidone.
- 20
- 25 However, problems arise in that the colour is affected by the time over which the blood sample is held in contact with the reagent pad, as well as the amount of blood in contact with the reagents. It is therefore customary for such tests to be carried out within a strictly monitored time schedule and the results are often of dubious value due to inaccuracies in observing the time schedule.
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Surprisingly, we have found that the proportion of the reagents to one another in the matrix affects the period over which a consistent colour is produced by the interaction of the blood with the reagent. If the proportions in the mixture lie within

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certain limits, the colour produced is sufficiently constant over a period of time for the need for strict adherence to a time schedule to be reduced.

5 SUMMARY OF THE INVENTION:

Accordingly, the present invention provides a blood test reagent mixture composition comprising the enzymes glucose oxidase and peroxidase and a chromogen which interacts with the  
10 hydrogen peroxide from the oxidation of the blood glucose by the glucose oxidase, characterised in that the glucose oxidase and the peroxidase are present in proportions which provide from 300 to 700 International Units (IUs) of glucose oxidase and at least 20 International Units of peroxidase and in that  
15 the chromogen is present in an amount which provides from 12 to 20 grams of active chromogen per 500,000 International Units of glucose oxidase. Preferably, the glucose oxidase is present in from 400 to 550 IUs per 27.5 to 32.5 IUs of peroxidase and the chromogen is o-tolidine which is present in an amount of from  
20 12 to 15 gs per 500,000 IUs of the glucose oxidase.

It is preferred that the reagent mixture be put up in a gel matrix, notably a gelatin matrix, which provides from 200 to 400 gs of the matrix on a dry weight basis per 500,000 IUs of  
25 the glucose oxidase.

It is particularly preferred that the reagent mixture/matrix be absorbed in a micro-porous membrane carrier.

30 The enzymatic reagents as used herein can be present in any suitable form, for example as the dry powdered active enzyme or as a precursor or addition product thereof which under the conditions of the test to be carried out produces an active enzyme in the reagent mixture. Thus, the enzymatic reagent can  
35 be an active enzyme, for example glucose oxidase or peroxidase, or a stabilised form thereof, for example an acetate or other

salt or adduct thereof, which releases the active enzyme when the reagent mixture is wetted.

For convenience, the invention will be described hereinafter in terms of a mixture of glucose oxidase and peroxidase as conventionally used in the assessment of glucose in a blood sample.

Similarly, the term chromogenic material is used herein to denote any material which develops a property upon interaction with one or more of the products produced when the enzymatic reagent reacts with the blood sample to be assessed. Thus, the term includes materials which develop ultraviolet fluorescence or other detectable but not visible properties. However, it is preferred that the chromogenic material be one which develop a colour within the visible spectrum, for example as when di-anisidine or o-tolidine reacts with the hydrogen peroxide released when glucose in blood interacts with the glucose oxidase in the reagent mixture. The chromogenic material can be used in the form of the active material or a precursor or adduct thereof, notably an inorganic acid salt thereof such as the hydrochloride or sulphate, which releases the active ingredient during the test.

For convenience, the invention will be described hereinafter in terms of o-tolidine as the chromogenic material.

The enzymatic reagent and chromogenic material are operatively associated with one another so that they can interact under the conditions of the test procedure. Typically, the reagent and the chromogenic material will be put up in physical admixture with one another. However, it is within the scope of the present invention to put up the reagent and chromogenic material in a two part form which is admixed immediately prior to use; or in a form in which the reaction products of the interaction of the enzyme reagent with the glucose in the blood

sample diffuse into a zone containing the chromogenic material to develop the colour therewith separately from the enzyme interaction zone. Thus, for example, the enzyme reagent can be concentrated at one end or one side of a reagent pad and the  
5 chromogenic material at the other end or side.

For convenience, the invention will be described hereinafter in terms of a pad of the reagent mixture containing the enzymatic reagent and the chromogenic material substantially uniformly  
10 distributed throughout the pad.

The reagent mixture may contain other materials as is customary, for example phosphate buffering agents, preservatives, anti-coagulants or surface active agents. Such  
15 other materials are typically inert to the material to be tested, the other constituents of the reagent mixture and the products of the reactions and interactions which occur during the test procedure. Such other constituents can be present in the amounts normally used in such reagent mixtures.

20 As stated above, we have found that if the enzymatic reagent and chromogenic material are present in the reagent mixture composition within specified proportions, the colour which the interactions between the material being assessed and the  
25 various components of the mixture is surprisingly stable and enables the colour to be observed over a wider period of time than hitherto. Thus, the enzymatic reagents will typically be present in proportions of from 400 to 600, notably 450 to 550, International Units (IUs) of glucose oxidase and at least 20  
30 IUs, typically about 27.5 to 35 IUs of peroxidase in the mixture. The chromogenic material will typically be present in an amount of from 12 to 17, notably from 13 to 16, grams per 500,000 IUs of the glucose oxidase. The optimum proportions within these ranges can be determined for any given case and a  
35 given carrier by simple trial and error tests.

As stated above, the reagent mixture is preferably put up in a matrix carrier medium so that the material to be assessed can penetrate to the enzyme reagent and the chromogenic material. The matrix can be provided by a natural gum, jelly or gel, for example a gelatin, agar agar, aspic or silica gel; or can be provided by a synthetic polymer gel, for example a cellulosic gel or a polyvinyllic resin gel. For convenience, the invention will be described hereinafter in terms of the use of a gelatin gel as the carrier matrix.

10 The gel matrix can carry the enzymatic reagent and chromogenic material substantially uniformly distributed throughout it. This is conveniently achieved by mixing the enzyme reagents into a premix of the gelling agent and the chromogenic material; and allowing the mixture to set in the desired form. Alternatively, the enzyme reagent and the chromogenic material can be admixed with a thixotropic gel carrier which is worked, for example by being stirred, to maintain it in the fluid state during mixing, but which is then allowed to set for storage and transport prior to use.

Alternatively, the matrix may contain the enzymatic reagent and chromogenic material non-uniformly distributed therein, as when a gel layer is formed which has a high concentration of the gel matrix in its upper layers to act a protective layer or coating for the reagent rich lower layers; or where the enzymatic reagent is located in a separate zone of the matrix from that containing the chromogenic material. In this case, the product from the interaction of the material being tested with one or more of the enzymatic reagents diffuses from the enzyme zone into the zone containing the chromogenic material to develop a colour as a separate stage in that zone.

For convenience, the invention will be described hereinafter in terms of a reagent mixture which is uniformly distributed throughout a gelatin matrix.

The matrix carrying the reagent mixture can be put up in a number of physical forms, for example as test strips or discs in which a pad of the matrix is applied to one face of the strip or disc and the colour resulting from the interaction of the material under assessment and the reagents and materials in the matrix is observed visually against the white background of the support strip or disc or against a separate reference background. Alternatively, the matrix can be put up in a series of zones through which the reagent mixture is distributed so that the interaction of the material to be assessed with the enzyme occurs in one zone and the product of that interaction diffuses to a second zone in which the colour reaction takes place. In a further alternative, the reagent mixture matrix can be absorbed or impregnated into the pores of a porous carrier to one face of which the material to be assessed is applied and the colour developing within the matrix is viewed from the opposite surface of the carrier.

For convenience, the invention will be described in terms of the use of a pad or disc of a micro-porous membrane which is impregnated with the reagent matrix.

In a conventional blood test reagent mixture, the gel matrix is a high molecular weight gelatin which is present in about 4% by dry weight. However, where the reagent mixture is to be absorbed into a micro-porous membrane, we prefer to use a low molecular weight gelatin, typically with a molecular weight in the range 20,000 to 50,000. Where such a gelatin is present in the amounts used hitherto, we have found that this results in a gel matrix which cannot be held satisfactorily within the pores of the membrane. On the other hand, we have found that if the gel content of the reagent mixture composition exceeds about 20% by dry weight, the gel inhibits the diffusion of reaction products through the membrane and hence development of a colour reflecting the true extent of the interactions which have occurred. We therefore prefer to provide the gel matrix



as a low molecular weight gelatin in an amount of from 250 to 325 gs by dry weight per 500,000 IUs of the glucose oxidase present in the reagent mixtrue.

- 5 The invention will now be illustrated by the following Example in which all parts and percentages are given by weight unless stated otherwise.

10 A first solution was made by stirring together at room temperature 300 mls of de-ionised water, 200 mls of 0.5 Molar sodium phosphate buffer to give a pH of 7, 100 mls of a 20% w/v solution of the surfactant Gantrez and 300 gs of dry powdered gelatin having a molecular weight in the range 25,000 to 40,000.

15 A second solution was prepared by stirring together at 60° C for one hour 300 mls of de-ionised water, 300 mls of methoxyethanol and 15 gs of o-tolidine hydrochloride or dianisidine hydrochloride.

20 The second solution was mixed dropwise with stirring into the first solution and the mixture stood for 1 hour at 60° C.

25 A third solution was made up by mixing 500,000 IUs of glucose oxidase and 30,000 IUs of peroxidase in a 0.1 Molar solution of the sodium phosphate buffer. This solution was mixed with stirring into the other mixed solutions and filtered through a 0.1 micrometre aperture filter.

30 The resultant solution was impregnated into a polysulfone resin sheet (0.2 to 0.4 mms thick and having an average pore diameter of 0.2 micrometres and an air permeability of 3 litres per minute per square centimetre at an applied pressure of 10 psig) to provide 5 IUs of glucose oxidase, 3 IUs of peroxidase, 0.2  
35 milligrams of o-tolidine and 4 milligrams of gelatin per square centimetre of the membrane.

By way of comparison, the same membrane was impregnated with a conventional reagent mixture to provide the conventional level of enzyme and chromogen per square centimetre.

- 5 Blood samples were applied to the faces of a number of 6 mm diameter discs cut from each of the membranes. With the reagent compositions of the invention, a blue colour developed after only 10 seconds. The hue and intensity of the colour became stable after about 30 to 40 seconds and remained stable  
10 for a further 30 to 40 seconds, thus allowing considerable latitude for the time to observe the colour. By way of comparison, the conventional formulations gave a colour which deepened in hue and intensity over 10 to 30 seconds after applying the blood sample, but which degenerated after a  
15 further 15 seconds, giving little or no latitude in the time for observing the true colour.

- From another aspect, the present invention provides a method for making a test reagent mixture of the invention, wherein the  
20 components of the mixture are admixed with one another to provide a substantially uniform mixture of the components.

- The invention further provides a method for making a test reagent mixture carried on a micro-porous carrier medium,  
25 wherein a fluid reagent mixture of the invention is applied to the carrier medium. Preferably, the mixture is applied by impregnating the medium, for example by padding a sheet of the carrier through a bath of the reagent mixture, and allowing the mixture to gel within the pores of the carrier. Preferably,  
30 the gelled mixture blinds the bores of the pores of the carrier so that rupture of blood or other cells due to capillary action by the pores is reduced. Discs or other shapes can be cut from the impregnated carrier for mounting on tests sticks having apertures therein or as the end walls of sample receivers so  
35 that the colour which develops in the carrier can be observed from the opposite side to that to which the blood is applied.

CLAIMS:

1. A blood test reagent mixture composition comprising the enzymes glucose oxidase and peroxidase and a chromogen which  
5 interacts with the hydrogen peroxide from the oxidation of the blood glucose by the glucose oxidase, characterised in that the glucose oxidase and the peroxidase are present in proportions which provide from 300 to 700 International Units (IUs) of  
10 peroxidase and in that the chromogen is present in an amount which provides from 12 to 20 grams of active chromogen per 500,000 International Units of glucose oxidase.
2. A test reagent mixture as claimed in claim 1,  
15 characterised in that the glucose oxidase is present in from 400 to 550 IUs per 27.5 to 32.5 IUs of peroxidase and the chromogen is o-tolidine which is present in an amount of from 12 to 17 gs per 500,000 IUs of the glucose oxidase.
- 20 3. A test reagent mixture as claimed in either of claims 1 or 2, characterised in that it is put up in a gel matrix.
4. A test reagent mixture as claimed in claim 3,  
25 characterised in that the gel matrix is a gelatin matrix, which provides from 200 to 400 gs of gelatin on a dry weight basis per 500,000 IUs of the glucose oxidase.
5. A test reagent mixture as claimed in any one of the preceding claims, characterised in that the reagent mixture is  
30 carried by a micro-porous membrane.
6. A test reagent mixture as claimed in either of claims 4 or 5, characterised in that the gelatin has a molecular weight in the range 20,000 to 50,000 and is present in an amount of from  
35 250 to 325 gs by dry weight per 500,000 IUs of the glucose oxidase.

7. A test reagent mixture according to claim 1, substantially as hereinbefore described in the Example.

8. A method for making a test reagent mixture as claimed in claim 1, characterised in that the components of the mixture are admixed with one another to provide a substantially uniform mixture of the components.

9. A method for making a test reagent mixture as claimed in claim 5, characterised in that a fluid reagent mixture as claimed in any one of claims 1 to 4 or claim 6 is impregnated into the pores of a micro-porous carrier membrane.

10. A method for testing blood samples, characterised in that it comprises applying blood to a test reagent mixture as claimed in claim 1 and observing the colour which develops.

11. A method as claimed in claim 10, characterised in that the reagent mixture is carried by a micro-porous carrier membrane and the blood is applied to one face of the membrane and the colour is observed from the opposite face of the membrane.

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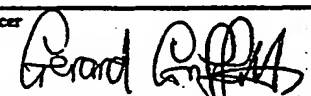
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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 92/01116

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| <b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup>  |  |  |
| According to International Patent Classification (IPC) or to both National Classification and IPC  |  |  |
| Int.Cl. 5 C12Q1/54; C12Q1/26; C12Q1/28   |  |  |
| <b>II. FIELDS SEARCHED</b>   |  |  |
| Minimum Documentation Searched <sup>7</sup>  |  |  |
| Classification System  | Classification Symbols   |  |
| Int.Cl. 5  | C12Q   |  |
| Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>   |  |  |
| <b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>  |  |  |
| Category <sup>10</sup>   | Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup> | Relevant to Claim No. <sup>13</sup>  |
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| A  | EP,A,0 016 947 (BOEHRINGER MANNHEIM GMBH.)<br>15 October 1980<br>see the whole document                        | 1-11   |
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| <b>IV. CERTIFICATION</b>   |  |  |
| Date of the Actual Completion of the International Search  |  | Date of Mailing of this International Search Report  |
| 02 OCTOBER 1992  |  | 11 5. 10. 92   |
| International Searching Authority<br>EUROPEAN PATENT OFFICE  |  | Signature of Authorized Officer<br>GRIFFITH G.  |

# ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9201116 SA 60990

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